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REVIEW

Proteome analysis of biological fluids from autoimmune-rheumatological disorders

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Autoimmune-rheumatological diseases are worldwide distributed disorders and represent a complex array of illnesses characterized by autoreactivity (reactivity against self-antigens) of T-B lymphocytes and by the synthesis of autoantibodies crucial for diagnosis (biomarkers). Yet, the effects of the autoimmune chronic inflammation on the infiltrated tissues and organs generally lead to profound tissue and organ damage with loss of function (i.e., lung, kidney, joints, exocrine glands). Although progresses have been made on the knowledge of these disorders, much still remains to be investigated on their pathogenesis and identification of new biomarkers useful in clinical practice. The rationale of using proteomics in autoimmune-rheumatological diseases has been the unmet need to collect, from biological fluids that are easily obtainable, a summary of the final biochemical events that represent the effects of the interplay between immune cells, mesenchymal cells and endothelial cells. Proteomic analysis of these fluids shows encouraging results and in this review, we addressed four major autoimmune-rheumatological diseases investigated through proteomic techniques and provide evidence-based data on the highlights obtained in systemic sclerosis, primary and secondary Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis.

Keywords:
Biomarkers / Rheumatoid arthritis / Sjogren's syndrome / Systemic lupus erythematosus / Systemic sclerosis

1 Introduction

Autoimmune-rheumatological disorders group includes Sjogren's syndrome (SS), systemic sclerosis (SSc), rheumathoid arthritis (RA), systemic lupus erythematosus (SLE), spondyloarthritis, dermatomyositis and vasculitides [1–4]. The autoimmune-rheumatological disorders are distributed worldwide with high social-economical impact (1% of the population), in particular women with a 3/1 gender ratio [3, 5, 6]. Previous studies on the pathogenesis of these disorders have shown that the cumulative effects of multiple environmental factors such as viral or bacterial infections and toxic agents in genetic predisposed hosts lead to the production of auto-reactive cellular and humoral immune responses against self-systems, which are responsible for acute and chronic organ damage, invalidating the patients and affecting their life-style (Fig. 1) [4, 7]. Although progresses have been made in the analysis of the pathogenesis of these disorders, a

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Abbreviations: Abs, antibodies; **BALF,** bronchoalveolar lavage fluid; **EC,** endothelial cell; **ILD,** interstitial lung disease; **JIA,** juvenile idiopathic arthritis; **LN,** lupus nephritis; **MALT,** mucosa-associated lymphoid tissue; **pSS,** primary SS; **RA,** rheumathoid arthritis; **SLE,** systemic lupus erythematosus; **SS,** Sjogren's syndrome; **sSS,** secondary SS; **SSc,** systemic sclerosis

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limited numbers of tools for clinical management and diagnosis are available until now.

The recent development of proteomic technologies for massive protein analysis has facilitated the study of biological fluids to identify potential new biomarkers useful for early diagnosis, clinical management and evaluation of treatments efficacy [8, 9]. The easier and less invasive available biological fluids are serum, plasma, urine, saliva, tears and bronchoalveolar lavage fluid (BALF) that contain an enormous amount of proteins or peptides involved in the regulation of different systems and possibly involved in the pathogenesis of these autoimmune-rheumatological disorders.

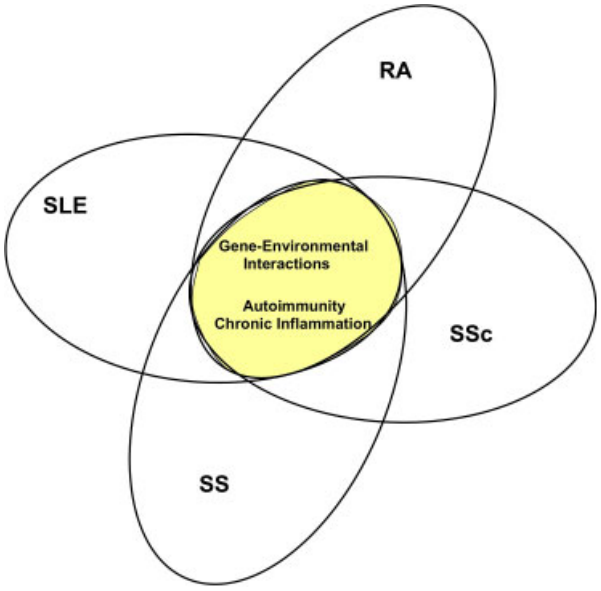

Figure 1. Schematic representation of the pathogenesis of the discussed autoimmune-rheumatological disorders: SS, SSc, RA and SLE. The cumulative effects of multiple environmental factors as viral or bacterial infections and toxic agents in genetic predisposed hosts lead to the production of auto-reactive cellular and humoral immune responses against self-systems, which are responsible for acute and chronic organ damage, invalidating the patients and affecting their life-style.

In the present review, we discuss the more significant proteomic studies on biological fluids in autoimmune-rheumatological disorders focusing on SS, SSc, RA and SLE (Table 1).

2 Proteomic studies in SS patients

SS is a chronic, inflammatory, autoimmune disease characterized by lymphocytic infiltration of the exocrine glands leading to either qualitatively altered and reduced secretion or absent glandular secretion, and by marked a B-cell hyperreactivity with (i) hypergammaglobulinemia and (ii) various serum autoantibodies such as the Abs against the Ro(SSA) and La(SSB) ribonucleoproteins, the rheumatoid factor, anti-α-fodrin (a cytoskeleton protein) and anti-M3R [10]. In addition, a possible severe complication is the development of B-cell lymphomas of the mucosa-associated lymphoid tissue (MALT) type [10]. Clinical presentation of SS disease is generally characterized by dry eyes (xerophthalmia) with possible associated keratoconjunctivitis sicca and dry mouth (xerostomia). SS disease may arise alone as primary SS (pSS) or as secondary SS (sSS) disorder in association with other well-established rheumatic disease as RA or SLE [10]. However, approximately 40% of the patients develop extraglandular manifestation as neurological, pulmonary and complications within the clinical follow-up

Table 1. Proteomic studies of biological fluids from patients with autoimmune-rheumathological disorders

	Biological markers used in clinical practice	Possible biomarkers for future use in clinical practice	Biological fluid analyzed	References
pSS	Anti-SSA/Ro Ab Anti-SSB/ La Ab, complement C3, C4	β2-Microglobulin, Cystatin C, Cystatin A, Actin, defensin-1, β-defensin-2, calgranulin B, albumin	Saliva Tears	[15–21]
SSc	Anti-ACA Anti-estractable nuclear Antigen-Scl70 Ab	SSA protein, complement factor H, mtDNAtopo 1, calgranulin B, Thymosins β Psoriasin S100, calgranulin A and B	Serum BALF Saliva	[41–49, 53, 54]
RA	RF Anti-CCP Abs, anti-MCV	CTL1; chain A of transthyretin, SSA, Apo IV, haptoglobin, Cp, Ig superfamily 22; Ig: immunoglobulin, CRP, cathepsin D, Thymosin β_4	Serum Plasma	[61, 63, 66–69, 74–78]
SLE	Anti-dsDNA Abs Complement C3,C4	INA Abs, I-IFN, transferrin, α1-acid-glycoprotein and lipocalin-type prostaglandin D-synthetase, hepcidin 20	Plasma Urine serum	[84, 85, 87, 88, 90–93, 99]

pSS, primary Sjogren's syndrome; SSc, systemic sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; Ab, antibody; anti-SSA Ab, autoantibodies agains epitopes on polypeptides associated with small RNAs called scYRNA situated mostly in the cytoplasmic compartment (70%) and few in the nuclear compartement (30%); anti-CCP Abs, antibodies to cyclic citrullinated protein; anti-dsDNA Abs, anti-nuclear antibodies; SSA protein, amyloid serum protein; CTL1, coactosin-like 1; Apo IV, apolipoprotein IV; Cp, cerulopasmin; ACA, anticentromere antibodies; INA Abs, anti-intermediate neurofilament α-intermexin; RF, rheumatoid factor; CRP, C-reactive protein; antiMCV, anti-mutated citrullinated vimentin antibodies.

[10]. SS prevalence is approximately 0.1–0.6% of the adult population [11].

Since the characteristic involvement of salivary and lachrymal glands in SS patients is clinically evident as xerostomia and xerophthalmia, the proteomic analysis of biological fluids as saliva or tears is important to identify new biomarkers involved in the pathogenesis of the disease and/or useful in clinical management of SS patients. In addition, saliva and tears are relatively simple biological fluids to collect and can mirror local and systemic pathological changes related to SS disease [8, 12].

Although studies on human salivary proteome in healthy subjects have been reported, much still remains to be investigated on the dynamic changes in saliva proteome among subjects, within the day in the same subject, among different salivary glands, and between salivary glands and crevicular fluid [13, 14].

Studies in SS patients have shown that xerostomia and xerophthalmia result from a loss of exocrine cell mass associated with neural dysregulation of glandular secretion due to the combination of the following factors: epithelial cells apoptosis, production of functional antagonistic autoantibodies as anti-M3R and infiltrating effector T cells [10]. However, the reduced tear and saliva flow rates are also associated with changes in their protein composition; thus, the proteomic analysis of these biological liquids represents an interesting tool in biomarker discovery [15]. In a literature review, we found five studies that investigated the saliva proteome from SS patients compared to normal controls [16–20], two studies investigated the compositions of tears from SS patients [15, 21], but none simultaneously evaluated saliva and tear proteome from the same SS patients.

Table 2 summarizes the more relevant information on the proteomic analysis of saliva from SS patients. All studies were carried out in pSS subjects compared to normal controls. In four studies, whole saliva proteome was analyzed and in 2 out of 5 studies, the authors have evaluated saliva from parotid glands, even though Hu et al. have

Table 2. Proteomic studies of saliva from SS patients

	Ryu HO et al. [16]	Giusti L et al. [17]	Peluso et al. [18]	Hu et al. [19]	Fleissig et al. [20]
Proteomic Approach	2-D DIGE	2-DE	HPLC	2-DE	2-DE
	SELDI-TOF	MALDI-TOF	ESI	MALDI-TOF LC-MS/MS microarray	ESI-MS/MS
Patients	41 Primary SS	12 Primary SS	9 Primary SS	10 Primary SS	6 Primary SS
Controls	15 Dry mouth 5 healthy	12 Healthy	10 Healthy	10 Healthy	8 Healthy
Biological liquid	Parotid saliva	Whole saliva	Whole saliva	Whole saliva Parotid saliva Submandibular/lingual	Whole saliva
Sample collection time	2 h after breakfast	Before breakfast	2 h after lunch	2 h breakfast	After 1 h since the last meal
Saliva stimulation	Citric acid	None	None pilocarpine	Chewing paraffin	None
Biomarkers: down-regulated proteins	PRP Amylase, Carbonic anhydrase VI	α-Amylase pr, Cystatin SN pr, Keratin 6-L, PIP	PF, IB-8a, IB-1, desR, II-2, desR, PRP1, 3, PC, desRPQ Statherin, 1P, desD1, SV2, des1-9, des1-10, P-B, des1-4 Cystatin C, S, S1, S2, SA, SN, histatin1-12	Carbonic anhydrase VI, Polymeric Ig R, Lysozyme C, Prolactin-inducible p, Von Ebner's gland p, Cystatin C, SN, D, S, SA, Calgranulin A	Polymeric Ig R, Vitamin D binding p, α-amylase
Biomarkers: up-regulated proteins	β2-Microglobulin, Lactoferrin, Ig κ light chain, Polymeric Ig R, Lysozyme C, Cystatin C	E-FABP, Actin, β-actin f, Leukocyte elastase inhibitor, GST, 5 not identified proteins	PB des1-4, Cystatin A, α-defensin-1, β-defensin-2	Calgranulin B, Psoriasin, Hemoglobin α, β chain, E-FABP, IGHG1, IGHM, α –enolase, α-amylase f, Fructose-bisphosphate aldolase A, Ig γ1-chain C-region, Carbonic anhydrase I, II, Caspase 14, Ig κ-chain C-region, β_2 microglobulin, Actin, Albumin f	Keratin I, II, Albumin, Actin, Fibrinogen β chain, Ig γ1-chain C-region, Calgranulin B, Calcium binding p

pr: precursor; Ig: immunoglobulin; R: receptor; p: protein; f: fragment; PIP: prolactine inducible protein; FABP: fatty acid binding protein; GST: glutathione S-transferase; IGHG: immunoglobulin heavy chain igG; IGHM: immunoglobulin heavy chain igM.

also analyzed saliva from submandibular and sublingual glands [19]. In these studies, the samples were collected after different time intervals, after breakfast or lunch, and in two studies a known saliva stimulator such as citric acid or chewing paraffin was used [16–20].

In SS patients, the proteomic data show that proteins of acinar origin, such as proline-rich proteins, histatins, and statherin, were reduced, while inflammatory acute-phase proteins, such as lactoferrin, fibrinogen and albumin, were increased compared to normal subjects [16–20]. Some proteins that were either two- to three-fold up- or down-regulated in SS patients compared to normal controls or exclusively present in SS saliva have been indicated as possible pSS biomarkers (Table 2). It is of interest to note that defensins, a class of immune-peptides involved in the innate immunity against bacteria and fungi, were found to be biomarkers of SS, being up-regulated in both saliva and tears from SS patients [18, 20, 22].

α and β defensins are a family of small cationic proteins, 3000–4000 Da, involved not only in the innate immune system against infectious microbes but also in adaptive immunity, inflammation and wound repair [13]. α-Defensins 1–4 are present in granules of neutrophils [13]. Recently, the presence of α-defensins 1–3 in saliva from normal subjects and low levels of α-defensin-4 in gingival in crevicular fluid from normal subjects has been detected by HPLC-ESI-MS [14]. In whole saliva from SS with both pSS and sSS form, Peluso et al. have reported increased α-defensin-1 levels in pSS patients compared to either normal controls or sSS subjects [18]. Since defensins play an important role in modulation of local anti-inflammatory response, we have proposed the increased amount of α-defensin-1 in pSS patients as result of periodontal disease that is common in pSS patients, suggesting a possible role of α-defensin-1 as a marker of oral inflammation in pSS patients [18].

β-Defensins 1–2 are mainly expressed by keratinocytes [23]. β-Defensin-1 is constitutively and heterogeneously expressed in epithelial cells and only poorly induced by cytokines. Otherwise, β-defensins-2 can be induced by local cytokine imbalance once a predominance of Th1 lymphocytes related cytokines such as the tumor necrosis factor-α, similarly to what observed in other oral inflammatory or infectious diseases [24]. Kawasaki et al. have reported a significant up-regulation of only β-defensin-2 gene in conjunctival epithelial cells from pSS patients [22]. Recently, we have found β-defensin-2 in saliva from pSS patients but not in healthy subjects, suggesting β-defensin-2 as marker of pro-inflammatory response in pSS patients, most likely related to the high levels of local cytokines such as TNFα [18, 25, 26].

The association of SS with marginal zone B-cell lymphomas is present in up to 5% of SS patients [27]. The salivary extranodal marginal zone B-cell lymphomas of MALT type are the most common lymphomas in SS [27]. Hu et al. have performed a transcriptomic and proteomic analysis of human parotid glands from patients with either pSS alone or associated with MALT lymphoma [28]. The authors proposed a panel of eight candidate genes (GRB2, ARHGDIB, CD40, proteasome subunit, aldolase A, peroxiredoxin 5, PARC, cyclophilin A) to distinguish pSS from pSS associated MALT form [28]. However, the clinical application of these markers is extremely limited and it would be of interest to carry out an integrated genomic and proteomic analysis of saliva and glandular tissues from these patients to validate the identified biomarkers [29].

However, the proteomic studies on saliva from SS patients still indicate the following: (i) the importance to develop a uniform protocol to collect saliva; (ii) the whole saliva as the more informative sample in SS patients compared to single gland-derived saliva; (iii) integration of different MS techniques to study low-molecular-weight proteins [30, 31].

3 Proteomic studies in SSc subjects

SSc is a multi-organ connective tissue disease characterized by microvascular abnormalities associated with skin and internal organ fibrosis. SSc prevalence is approximately 0.001–0.004 % of the adult population [7, 32]. To date, no effective treatment is available, probably because the etiopathogenesis of SSc is not completely clarified. SSc is characterized by three hallmarks: microvascular abnormalities, autoimmune system activation and tissue fibrosis [33]. It seems that the primary event in the pathogenesis of the disease is the endothelial cell (EC) damage with infiltration of mononuclear inflammatory cells and increased extracellular collagen deposition [34]. Several evidence suggests that environmental agents lead to the production of an autoimmune response in a genetically predisposed host, but the cause of the higher host susceptibility remains to be discovered. Abs against different cellular and intracellular antigens are detected in 80–90% of serum from SSc patients; autoantibodies to several nuclear and nucleolar antigens or other autoantobodies such as anti-EC Abs (Table 1). It has been shown that anti-EC Abs are capable of promoting the leukocyte adhesion to ECs and also of inducing the death of these cells. Other hypothesis, not mutually exclusive, suggests that oxygen-free radicals and cytokines may play a central role in the pathogenesis of SSc. The reactive oxygen species produced during chronic inflammatory response, further amplify the vascular damage in SSc patients, resulting in EC dysfunction and fibroblast proliferation [35]. In addition, the transforming growth factor-β and the connective tissue growth factor activate fibroblasts and contribute to fibrosis by increasing the extracellular matrix deposition [36].

In SSc patients, the lung involvement is the leading cause of morbidity and mortality [37], but the variability of lung damage and the rate of progression vary between SSc patients. The BALF analysis is essential to diagnose alveo-

litis that characterize a subset of SSc patients [37–39]. Thus, BALF analysis is actually considered a suitable method just to evaluate the presence of lower respiratory tract infections in clinical setting or to obtain material for research purposes [40, 41].

Several papers on proteomic analysis of BALF, saliva and serum in SSc patients have been recently reported [41–48], allowing an insight on proteins that are potentially crucial to the biology and the development of fibrotic and vascular damage in SSc subjects (Table 1).

4 BALF and saliva proteomic studies in SSc patients

To date, the studies on BALF proteome in SSc are not conclusive in demonstrating a SSc-specific protein pattern [41–44]. Rottoli et al. have conducted a 2-D analysis of BALF from SSc patients with interstitial lung disease (ILD) [41, 42]. The authors have reported quantitative rather than qualitative differences in protein profile in the comparison between SSc patients with lung involvement and patients with other ILD forms, such as idiopathic pulmonary fibrosis or sarcoidosis [41]. The differently expressed proteins belong to the classes of proteases/antiproteases, coagulation system, cytokines, antioxidant proteins and calcium binding proteins. The study highlighted that plasma-derived and locally produced proteins have intermediate levels in BALF from SSc subjects when compared to that from patients with sarcoidosis or idiopathic pulmonary fibrosis.

In another study, Fietta AM et al. compared BALF from SSc patients with and without lung disease by 2-D with MALDI-TOF and HPLC-MS analysis [43]. The authors found nine differently expressed proteins in BALF. Among these proteins, a fragment of mtDNAtopo 1 was found only in BALF from SSc patients with ILD, while glutathione S-transferase P and cystatin SN were detectable only in SSc patients without lung disease. In addition, increased levels of calgranulin B, a molecule promoting extracellular matrix deposition and lung fibrosis [44], were found in BALF from SSc patients with ILD compared to SSc patients without lung involvement, similarly to what observed in BALF from patients with idiopathic pulmonary fibrosis [41–44]. The major limitation of these studies are (i) the small number of patients, (ii) the absence of control subjects and (iii) the lack of sensitivity of 2-D MALDI-TOF approach in identifying low molecular weight proteins. We have recently reported preliminary data on BALF from SSc patients, using reverse phase-HPLC coupled to a quadrupole ion-trap MS via an ESI analysis to identify small proteins and peptides and we found thymosins β in BALF from SSc subjects [49]. Thymosins β are peptides involved in cytoskeleton rearrangement and transcription of signaling molecules [50]. Since recent studies have indicated a functional role of these peptides in angiogenesis, fibrosis and reparative process [51, 52], the identification of β thymosins in BALF from SSc patients might suggest their possible involvement in the pathogenesis of SSc disease. In fact, we found increased levels of thymosin β_4 in SSc patients with ILD and interestingly, the sulfoxidated form of thymosin β_4, derived from the oxidation of a methionine residue, was found to be a possible biomarker of lung inflammation and oxidative stress in our SSc cohort [49]. These data are in agreement with a previous study reporting increased carbonylated proteins in BALF from SSc patients as a marker of oxidative and inflammatory damage in SSc lung [42].

A proteomic study on fibroblasts from BALF of patients with SSc by 2-DE in combination with MS has been reported [45]. In particular, 24 differently expressed protein spots had been identified, including cytoskeletal proteins (such as vimentin, tropomyosin and actin associated proteins) and proteins involved in redox imbalance (such as disulfide isomerase and glutathione S-transferase), which may contribute to EC damage, fibroblast trafficking and inflammatory response in SSc patients [45]. In another study, on saliva from SSc patients with a moderate–severe decrease of diffusion lung capacity for carbon monoxide and with the evidence of psoriasin has been described [48]. Psoriasin is a member of S100 protein family and is a potent chemotactic inflammatory protein for neutrophils and $CD4^+$ T lymphocytes, and it might be interesting to evaluate it in other biological fluids from SSc patients to better understand its possible role in the pathogenesis of this disorder.

Thus, the proteomic approach in the analysis of BALF or saliva from SSc patients with lung involvement might help to characterize the complex alveolar microenvironment, which is important for insights into pathogenetic mechanisms of ILDs, and to identify groups of disease related proteins including diagnostic and prognostic markers and new targets of therapy.

5 Proteomic analysis of serum protein profile from SSc patients

In sera from SSc patients, Brandwein et al. have reported high levels of amyloid-related serum protein that is involved in chemotaxis of inflammatory cells, modulation of proinflammatory cytokines and EC proliferation [53]. Recently, we have carried out a comparative proteomic analysis of sera from SSc patients and we identified 14 differentially expressed proteins that are mainly involved in EC protection and immune response [54]. In particular, we found increased concentrations of complement factor H in SSc subjects compared to healthy controls. Factor H is an important regulator of the alternative complement pathway, which normally protects self-cellular surface from complement cascade. Interestingly, we documented a defective capacity of factor H to bind ECs and to protect them from complement-mediated damage. An aberrant expression of complement regulatory proteins has been already demonstrated in the endothelium of patients with SSc and it has

been suggested that an inadequate protection from complement activation on cellular surface may be very important in the early phase of SSc disease. In fact, the recruitment of circulating fibroblasts progenitor cells and their activation into the tissue are events that may be facilitated by the microvascular dysfunction allowing the development of fibrotic and vascular damage characteristic of SSc disease [54, 55].

6 RA

RA is a chronic autoimmune inflammatory disease primarily involving the diarthrodial joints, such as hands, feet or knees [56]. However, the systemic nature of RA can lead to the involvement of several organs (i.e., lungs, vessels, nerves, bones) with the development of various comorbidities [57]. Inflammation usually starts in the synovial membrane where inflammatory-immune cells migrate from vessels. The inflamed synovial tissue is a complex mix of cells (fibroblasts, macrophages, lymphoid cells, dendritic cells, mast cells) that invades and destroys the cartilage through the synthesis of several cytokines and matrix metalloproteinases, resulting in erosion and destruction of the joints [57, 58]. RA prevalence rate is approximately 0.5–1% of the adult population [59, 60].

Proteomics have been used to define the biochemical/immunological changes in serum plasma proteome and the effects of biologic therapies (Table 3).

Liao et al. used 2-D LC-MS/MS in the analysis of synovial fluid and serum protein profile from RA patients with and without bone erosions [61]. In the more severe RA form, associated with erosive disease, in synovial fluid they found increased levels of C-reactive protein and calgranulin A, B, C that are potent pro-inflammatory molecules with chemotactic properties [62]. Recently, Jin et al. have reported the protein expression profile of plasma and sera from RA patients compared to healthy controls analyzed by 2-DE followed by MALDI-TOF-MS. The comparative proteomic analysis allowed the identification of 17 differently expressed proteins mainly related to inflammatory response [63]. Between the identified proteins in sera from RA patients, the authors focused on the small protein coactosin-like 1 that binds filamentous actin or 5-lipoxygenase, an enzyme involved in leukotriene biosynthesis in leukocytes [64, 65]. The authors further found a significant association of the c.484G > A polymorphism of protein coactosin-like 1 with the levels of anti-cyclic citrullinated peptide Ab, a biomarker used in diagnosis and clinical follow-up of RA patients, suggesting a possible increased susceptibility of subjects with protein coactosin-like 1 polymorphism for development of RA [63].

Table 3. Proteomic studies on biological fluids from rheumatoid arthritis (RA) patients and effects of biological therapies

Rheumathoid arthritis (RA)	Biological fluid studied	Proteomic approach	Differentially expressed proteins
Liao et al. 2004	Serum Synovial fluid	2-D-LC-MS/MS	C-reactive protein, calgranulin A, B, C
Jin et al. 2009	Serum Plasma	2-DE & MALDI-TOF MS/MS	COLT1, SAA1
Li et al. 2010	Serum		
Long et al. 2010	Serum	MB & MALDI-TOF MS	3 peaks at 2490; 5910.07; 6436.73 *m/z*
de Seney et al. 2005	Serum	SELDI-TOF MS	MRP-8
Serada et al. 2010	Serum	iTRAQ-MS validated by ELISA	LRG, actin, thymosin β, vimentin, tubulin, calgranulin A, B, C
Zheng et al. 2009	Plasma	RP-HPLC & Linear ion trap-FT	CRP, SAA, Actin, Thymosin β4, Talin 1, Calgranulin a,b,c
Goëb et al. 2009	Serum	2-DE & MALDI-TOF MS/MS	FUSE-BP
Steendam et al. 2010	Serum Synovial fluid	1/2-DE & LC-MS/MS	Citrullinated fibrinogen β, citrullinated vimentin
RA and Infliximab Dwivedi et al. 2009	Serum	iTRAQ-LC-MS	TNF-α and NF-kB related proteins, C-reactive protein
RA and Infliximab Trocme et al. 2009	Plasma	SELDI-TOF MS/MS	PF4, Apolipoprotein
RA and Infliximab Sekigawa et al. 2008	Serum Plasma	2-DE-LC-MS/MS	TNF-α and NF-kB related proteins
RA and Rituximab Fabre et al. 2009	Serum	PBAT	C-reactive protein, IL-6

PBAT: protein biochip array technology; COLT1: Coactosin like 1; SAA: serum amyloid protein; CRP: C reactive protein; FUSE-BP: far upstream element-binding proteins PF4: Platelet factor 4; MCP-1: monocyte chemoattractant protein-1; EGF: epidermal growth factor; IL6: interleukin 6; MRP8: myeloid related protein-8; ELISA: enzyme linked immunoassorbent assay.

In another proteomic study, sera from RA patients were studied by 2-D and MS analysis [66]. Eight proteins either over or underexpressed in sera of RA patients were identified. Among them, chain A of transthyretin was underexpressed, while serum amyloid A protein, apolipoprotein A-IV, haptoglobin 2, ceruloplasmin, immunoglobulin superfamily 22 and HT016 were over-expressed [66].

To study the low-abundant proteins in serum from RA patients, Long et al. have analyzed profiling serum proteins using magnetic bead-based separation followed by MALDI-TOF-MS analysis [67]. A pattern based on three mass/charge peaks (m/z 2490, 5910.07, 6436.73) discriminated patients with RA from healthy controls with a sensitivity and specificity of 87.5 and 96.7%, suggesting the possible use of this proteomic approach for early diagnosis of RA.

Using SELDI-TOF-MS to evaluate the low-molecular-weight proteins, de Seny et al. [68] examined serum samples from 34 RA patients compared to two control groups: a group of patients with other inflammatory diseases, such psoriatic arthritis or Crohn's disease and a group of healthy subjects. Two chip-arrays were used: CM10 as cation exchange surface and H4 as hydrophobic surface. The most discriminant mass/charge m/z peaks were highlighted: m/z values of 2924 (RA versus controls on H4 arrays), 10 832 and 11 632 (RA versus controls on CM10 arrays). Based on these evidences the 10 832 peak was considered as possible myeloid related protein-8, which is up-regulated in different autoimmune disorders and it has been shown to be functionally connected with autoreactive lymphocytes [61, 69]. They validated their hypothesis by immunoblot analysis and showed the association between the 10 832 peak and anti-cyclic citrullinated peptide Ab in sera from RA patients, supporting the proteomic analysis to identify possible biomarkers of inflammatory diseases.

Goëb et al. used a different strategy to identify new autoantibodies in early RA useful as new biomarkers in clinical management. The authors analyzed serum protein by immunoblot using HL-60 cell extract and identified by MALDI-TOF approach. Phosphoglycerate kinase 1 and the stress-induced phosphoprotein 1–2 were identified as new antigens in sera from RA patients. Stress-induced phosphoprotein 1–2 is a trans actin protein that may stimulate the promoter activity of the pro-oncogen *c-myc*, the expression of which has been shown to be increased in sinovial tissue fibroblast from RA subjects. Thus, citrullination of stress-induced phosphoprotein 1–2 through dysregulation of *c-myc* might participate in RA synovial hyperplasia [70–73]. In addition, the authors suggest that autoantibodies recognizing citrullinated peptide from stress-induced phosphoprotein 1–2 may increase the sensitivity of the currently used anti-cyclic citrullinated peptide test [74].

Recently, van Steendam et al. have analyzed serum and synovial fluid (SF) of RA patients compared to normal controls. Protein analysis was carried out combining detection of citrullinated proteins by Western blot and protein identification by LC-MS/MS. The authors showed that citrullinated fibrinogen β and citrullinated vimentin were the prominent citrullinated antigens in immunocomplex from SF of RA patients with elevated levels of citrullinated proteins, suggesting that citrullinated vimentin might play a role in sustaining active RA [76].

Along the lane of distinguishing between RA patients and controls, a quantitative proteomic approach that used iTRAQ followed by MS analysis further validated by enzyme-linked immunoassorbent assay showed that among 326 proteins identified by proteomic analysis, increased serum levels of leucine-rich α-2 glycoprotein was present in RA patients [75].

Relative differences in the protein classifications were observed in RA human plasma samples compared with controls: structural proteins, immuno-related proteins, protease inhibitors, coagulation proteins, transport proteins and apolipoproteins. While some of these proteins/peptides have been previously reported to be associated with RA disease such as calgranulin A, B, C and C-reactive protein, several others were newly identified, such as thymosin 4, actin, tubulin and vimentin [77].

The recent development of biological drug therapies against known targets involved in inflammatory response such as the monoclonal Ab against the tumor necrosis factor-α (infliximab) or the chimeric monoclonal Ab against the B lymphocytes surface protein CD20 (rituximab) has dramatically changed the clinical management of RA patients. Proteomic approaches have been used to find new biological indicators of clinical response to these new treatments possibly useful in clinical practice (Table 3, grey area) [78–81]. Dwivedi et al. have analyzed serum proteome from RA patients treated with infliximab after depletion of the most abundant proteins followed by labeling with iTRAQ to carry out a quantitative analysis [79]. They identified an average of 373 distinct proteins per patient with greater than 95% confidence. In sera from 3 out of 10 RA patients studied, they found significant changes in 39 serum proteins following the treatment. The large part of these proteins were directly or indirectly regulated by the tumor necrosis factor-α and nuclear factor-B, with acute-phase proteins being uniformly down-regulated. In another proteomic study, plasma from RA patients treated with infliximab were analyzed by SELDI-TOF-MS technology on two proteo-chip: SAX2 as anion exchange surface and IMAC3-Ni, as nickel affinity array, combined with biomarker characterization by either metal affinity chromatography or ammonium sulfate precipitation followed by MALDI-TOF analysis [80]. The authors identified six proteins as possible biomarkers and suggested apolipoprotein A1 as predictive of positive response to infliximab and platelet factor 4 as marker of RA non-responders to infliximab.

Sekigawa et al. investigated by 2-D LC-MS/MS approach sera and plasma from RA patients ($n = 10$) treated with infliximab compared to normal controls [81]. They observed

changes in protein expression related to tumor necrosis factor-α and nuclear factor-κB similarly to Dwivedi et al. [79].

Proteomic analysis by proteo biochip array technology was used to study the effects of rituximab on sera from RA patients [78]. The authors found significantly higher serum levels of pro-inflammatory cytokines, monocyte chemoattractant protein-1 and epidermal growth factor in responders than in non-responders RA patients and a significant decrease of C-reactive protein and IL-6 levels in the responder RA patients after 3 months of treatment with rituximab, suggesting that the proteomic analysis might be an interesting tool to identify biomarkers helpful in discriminating responder from non-responder RA patients.

These studies show that the analysis of serum plasma despite the wide amount of proteins by proteomic approach might allow the early diagnosis of complex autoimmune diseases such as RA, the identification of biomarkers useful for either clinical follow-up or identification of RA patients responders to biological treatments.

7 SLE

SLE is a systemic autoimmune disease characterized by a large variety of clinical manifestations associated with autoantibodies against nuclear autoantigens as well as cytoplasmic and circulating proteins (Table 1) [82]. These autoantibodies in the presence of abnormally activated acquired and innate immunity are responsible for cytotoxic and immune complex-mediated organ damages involving skin, kidney, brain, lung, joint and serosal tissues [82, 83]. The prevalence of SLE is in the order of 20–100 cases per 100 000 individuals, but varies by gender, ethnicity, socio-economic status, and genetic and environmental backgrounds [82, 83]. Although various Abs and different cytokines have been proposed for the pathogenesis of SLE, their role in the disease remains often to be elucidated.

Recently, Lu et al. have detected high titers of anti-intermediate neurofilament α-intermexin Abs in serum and cerebrospinal fluid of patients with neuropsychiatric systemic-SLE, which seem to be very important in the development of the disease [84]. In fact, they have demonstrated that mice, previously immunized with recombinant intermediate neurofilament α-intermexin, exhibit cognitive deficits that raise neuropsychiatric systemic-SLE symptoms and develop cortical lesions with related neuron apoptosis. In another work, Bauer et al. have shown that interferon regulates chemokine expression in serum of SLE patients [85]. High levels of interferon had been already documented in serum of SLE patients, especially in subjects with active lupus [86]. Using immunoassay, the authors have identified 30 type I-interferon dysregulated proteins, whose levels correlate with clinical and laboratory measures of disease activity. This group of patients present up-regulated proteins including cytokines IL-5, IL-6, IL-15, IL-18, brain derived neurotrophic factor and glial cell line-derived neurotrophic factor, cytokine receptors IL-2SRA and TGF-B-RIII, metalloproteinase 7 and adhesion molecule such as the intracellular adhesion molecule-5. Down-regulated proteins have been also documented, such as interferon-Ω, angiotensin converting enzyme, chemokine CCL20, growth factor FGF-2, growth factor receptors FGF-R3 and platelet-derived growth factor type A receptor. Moreover, Li et al. have highlighted Abs class-switch from IgM to the more pathogenic IgG class in serum of SLE patients with high levels of α-interferon [87].

Haptoglobin is another acute-phase protein that resulted increased in plasma of SLE patients with high activity of disease when compared with either healthy controls or SLE patients with low SLEDAI clinical score of disease activity (0–5). Increased levels of haptoglobin type 2-2 and haptoglobin 2-1S and decreased levels of haptoglobin 2-1F are the major peptide differences detected in plasma from these patients by proteomic approach [88]. It has been postulated that haptoglobin 2-2 phenotype is associated with increased Abs production and lower hemoglobin-binding capacity that may result in renal damage [89]. Proteomic analysis of urine from SLE patients has also identified several biomarkers with possible predictive power in disease flares. Suzuki et al. have investigated the urinary protein profile in 32 paediatric patients with SLE and 11 subjects with juvenile idiopathic arthritis (JIA) [90]. They have not found significant differences between SLE patients without nephritis and JIA patients, which have been used as controls since the JIA patients have inflammatory joint involvement related to autoimmune genesis but without the specific autoantibodies and the clinical presentation of SLE (Table 1). Conversely, they have detected eight protein spots significantly greater expressed in subjects with lupus nephritis (LN) compared with JIA controls and SLE patients without nephritis. By MALDI-TOF-MS analysis, they identified these molecules as transferrin, ceruloplasmin, α1-acid-glycoprotein, lipocalin-type prostaglandin D-synthetase, albumin and albumin-related fragments that strongly correlate with the renal SLE disease activity. It is interesting to note that urinary levels of transferrin, α1-acid-glycoprotein and lipocalin-type prostaglandin D-synthetase are increased few months before the clinical presentation of renal damage [91]. Mosley et al. have analyzed by SELDI-TOF-MS urine samples of 26 active LN patients and have compared the results with the urinary protein profile of 49 inactive LN subjects [92]. They have found two protein ion peaks with m/z of 3340 and 3980 that are strongly associated with active LN that could represent a specific assay to monitor patients with renal disease without invasive procedures, such as renal biopsies.

In a recent study, Zhang et al. have demonstrated that hepcidin isoforms are differentially expressed in the urine of SLE patients during a renal flare cycle [93]. In particular, hepcidin 20 is increased 4 months before renal flare and returned to baseline 4 months after the flare; hepcidin 25 is

decreased at renal flare and returned to baseline 4 months after the flare. Hepcidine is a small peptide that is involved in the iron metabolism and is up-regulated during a chronic inflammatory processes [94, 95]. Hepcidin expression is regulated by IL-6 and tumor necrosis factor-α [96, 97] and both cytokines are known to be involved in LN kidney disease [98].

Finally, Varghese et al. have examined urine from of 32 patients with LN, focal segmental glomerulosclerosis, membranous nephropathy or diabetic nephropathy, using 2-DE and MALDI-TOF/TOF or LTQ-Orbitrap MS analysis [99]. They have identified 11 peptides that may be potential urine biomarkers for the diagnosis of the major renal diseases: orosomucoid, transferrin, α-1 microglobulin, zinc α-2 glycoprotein, α-1 antitrypsin, complement factor B, haptoglobin, transthyretin, plasma retinol binding protein, albumin, and hemopexin. Moreover, they have generated urine protein patterns for the most common glomerular diseases by an artificial neural network analysis, but these data must be validated in a larger cohort of patients and with a more reproducible assay, as suggested by the authors themselves.

8 Concluding remarks

High-throughput MS approaches coupled to different separation techniques (i.e., HPLC and 2-D) have been used to study various human fluids from patients with auto-immune-rheumatologic diseases with the aim to enhance our understanding of disease pathogenesis and to identify biomarkers for non-invasive disease diagnosis, staging or monitoring. Several factors make difficult to define a panel of markers that can collectively enhance the sensitivity and specificity for the non-invasive diagnosis of rheumatologic diseases, such as SS, SSc alveolitis, RA or SLE. The most important limits are (i) the lack of a comprehensive proteomic analysis of saliva, BALF and urine of healthy subjects; (ii) the lack of uniform protocols to collect and prepare these samples for proteomic analysis; (iii) the high grade of posttranslational modification of the proteins in these fluids; and (iv) the difficulty to study low molecular weight proteins and peptides expressed locally and better reflecting the pathogenesis of the organ involvement. Until now, the proteomic studies have identified new interesting proteins in biological fluids from patients with auto-immune-rheumatologic disorders, opening new scenario on both pathogenesis of these diseases and their clinical management.

Future studies should be designed on a large number of patients and with standardized protocols most likely through an international network involving specialized clinical and proteomic centers working together in the development of tools suitable for routine clinical use and for progress in knowledge of the pathogenesis of these invalidating, chronic disorders.

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